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Original Contribution

Asymmetric dimethylarginine exacerbates Aβ-induced toxicity and oxidative stress in human cell and *Caenorhabditis elegans* models of Alzheimer disease

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ABSTRACT

Growing evidence suggests a strong association between cardiovascular risk factors and incidence of Alzheimer disease (AD). Asymmetric dimethylarginine (ADMA), the endogenous nitric oxide synthase inhibitor, has been identified as an independent cardiovascular risk factor and is also increased in plasma of patients with AD. However, whether ADMA is involved in the pathogenesis of AD is unknown. In this study, we found that ADMA content was increased in a transgenic *Caenorhabditis elegans* β-amyloid (Aβ) overexpression model, strain CL2006, and in human SH-SY5Y cells overexpressing the Swedish mutant form of human Aβ precursor protein (APPsw). Moreover, ADMA treatment exacerbated Aβ-induced paralysis and oxidative stress in CL2006 worms and further elevated oxidative stress and Aβ secretion in APPsw cells. Knockdown of type 1 protein arginine *N*-methyltransferase to reduce ADMA production failed to show a protective effect against Aβ toxicity, but resulted in more paralysis in CL2006 worms as well as increased oxidative stress and Aβ secretion in APPsw cells. However, overexpression of dimethylarginine dimethylaminohydrolase 1 (DDAH1) to promote ADMA degradation significantly attenuated oxidative stress and Aβ secretion in APPsw cells. Collectively, our data support the hypothesis that elevated ADMA contributes to the pathogenesis of AD. Our findings suggest that strategies to increase DDAH1 activity in neuronal cells may be a novel approach to attenuating AD development.

Alzheimer disease (AD) is one of the most common progressive and neurodegenerative disorders affecting the elderly. AD is characterized by progressive loss of neurons, cognitive decline, and two defining histopathologies: extracellular amyloid plaques and intracellular tangles composed primarily of β-amyloid (Aβ) peptide and hyperphosphorylated tau, respectively [1]. Although the exact etiology of AD remains elusive, there is a strong association between cardiovascular disease (CVD) and the incidence of cognitive decline and Alzheimer disease [2]. Individuals with subclinical CVD are also at higher risk for dementia and AD. The link between cardiovascular risk factors and AD has yet to be identified; however, a common feature of AD and CVD is endothelial dysfunction, specifically, decreased bioavailability of nitric oxide (NO) [3].

Asymmetric dimethylarginine (ADMA) is an endogenous inhibitor of nitric oxide synthesis. It is derived from methylation of arginine residues by type I protein arginine methyltransferase (such as PRMT1 and PRMT3) and subsequent proteolysis [4]. Plasma ADMA levels are increased in patients with AD [5,6] and cardiovascular disease, such as heart failure [7], hypertension [7,8], diabetes [9], and atherosclerosis [10], and increased ADMA is a strong and independent predictor of all-cause mortality in the community [11] and the strongest predictor of mortality in patients with congestive heart failure [12].

Recent studies have demonstrated that plasma ADMA is increased in AD patients and may contribute to the pathogenesis of AD [5,6]. In addition, neuronal NO synthase (nNOS)-derived NO production plays an important role in synaptic events involved in learning and memory [13], so the endogenous NOS inhibitors ADMA and N^G-methyl-L-arginine may disrupt processes by attenuating nNOS-derived NO production [14]. In AD patients, there is an inverse correlation between the concentration of NO in the cerebrospinal fluid (CSF) and the degree of cognitive impairment,

Abbreviations: AD, Alzheimer disease; ADMA, asymmetric dimethylarginine; APP, β-amyloid precursor protein; APPsw, Swedish mutant form of human β-amyloid precursor protein; Aβ, β-amyloid; CSF, cerebrospinal fluid; DDAH, dimethylarginine dimethylaminohydrolase; ERK, extracellular signal-regulated kinase; GSH, reduced glutathione; GSSG, oxidized glutathione; HSP60, heat shock protein 60; MAPK, mitogen-activated protein kinase; NGM, nematode growth medium; NOS, nitric oxide synthase; PRMT1, type 1 protein arginine *N*-methyltransferase; RNAi, RNA interference; ROS, reactive oxygen species

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suggesting a neuroprotective effect of NO in AD [15]. Whereas the levels of CSF ADMA in AD patients appear either reduced [5,16] or unchanged [17,18], plasma ADMA concentrations appear to be increased in AD patients [5,6]. Clinical results of older community-dwelling adults suggested that higher plasma ADMA was significantly correlated with memory impairment [19]. However, the effect of increased ADMA on the pathogenesis of AD is still unknown.

The transgenic *Caenorhabditis elegans* strain CL2006, which constitutively expresses Aβ under a muscle-specific promoter, is an ideal in vivo model to investigate Aβ toxicity-related behavior disorder and oxidative stress [20], whereas the human SH-SY5Y cell line stably overexpressing the Swedish mutant of human amyloid-β precursor protein (APPsw) is a useful in vitro model to study Aβ production. In the present study, we examined the effects of ADMA and its metabolism on Aβ toxicity and oxidative stress in these two models.

Materials and methods

An extended materials and methods section can be found in the online supplementary data.

Worm strains and maintenance

The wild-type Bristol N2 strain and the transgenic AD-associated nematode strain CL2006 (dvIs2[unc-54: human β-amyloid 1–42; pRF4]) were obtained from the *Caenorhabditis* Genetics Center (University of Minnesota). Maintenance of all strains was routinely performed at 20 °C and 40% relative humidity on nematode growth medium (NGM) plates with *Escherichia coli* strain OP50 as a food source as previous reported [21].

Paralysis assay

Synchronized hermaphroditic worm populations were transferred to plates with floxuridine (100 mg/L). On the first day of adulthood, 240 worms were placed on six plates. A live *E. coli* OP50 suspension with vehicle or ADMA (1 μM) was placed on the surface of the NGM plates (three plates for each experiment). The worms were tested for paralysis by tapping their noses with a platinum wire. Worms that moved their nose but failed to move their body were scored as "paralyzed." To avoid scoring old worms as paralyzed, the paralysis assay was terminated on day 12 of adulthood [22]. The final results were collected from three independent experiments.

Reactive oxygen species (ROS) measurement in C. elegans

The level of ROS in *C. elegans* was measured by the 2′, 7′-dichlorofluorescin diacetate (DCFH-DA) method as previously reported [21]. Worms were washed off the plates with cold M9 buffer. Then the worms were resuspended in M9 buffer with 100 μM DCFH-DA and allowed to equilibrate to room temperature. One hundred worms were transferred into each well of a black 96-well plate. ROS-associated fluorescence levels were measured every 10 min for 90 min in a Thermo Labsystems Fluoroskan Ascent microplate reader at excitation/emission wavelengths of 485 and 520 nm, respectively. Worms in the M9 buffer without DCFH-DA were used as negative controls. After the fluorescence was measured in the microplate reader, the worms were transferred to agar-padded slides (2% agarose) and sealed with a coverslip. 2′,7′-Dichlorofluorescein (DCF) fluorescence in live worms (15–20 worms in each group) was imaged using confocal microscopy (Olympus FV1000, Tokyo, Japan) with an excitation/emission filter (480/520 nm).

RNA interference

RNAi was performed in *C. elegans* by feeding the worms with dsRNA-containing bacteria. Briefly, gravid adult worms were treated using the NaClO method for progeny synchronization. After 12–24 h, the L1 larvae were grown on RNAi plates seeded with HT115 (DE3) bacteria expressing dsRNA of the corresponding target gene. After reaching the young adult stage, the worms were maintained on the RNAi plates until assays were performed. The control and PRMT1 RNAi constructs were kindly provided by Professor Chonglin Yang (Institute of Genetics and Developmental Biology, CAS).

Cell cultures and treatments

Human neuroblastoma SH-SY5Y cells stably expressing human APPsw or the empty vector pCLNCXv.2 (*neo*) were cultured as previous reported [23]. The pLKO.1–TRC cloning vector (Addgene Plasmid 10878) was used to construct clone short hairpin (sh) RNA against human dimethylarginine dimethylaminohydrolase 1 (pLKO.1–DDAH1, target sequence is TGAGCATGTCTGAACTGGAAA). PLKO.1–scramble shRNA was used as control. After being plated, the cells were treated with ADMA, or infected with human DDAH1 adenovirus/shRNA, or transfected with siRNA against PRMT1 (sense, CCAUCGACCUGGACUUCAATT; antisense, UUGAAGUCCAGGUCGAUGGTT) for the indicated time. Then cell viability, intracellular ROS/NO levels, and Aβ secretion were measured as described in the supplementary material.

Western blotting

The cells were washed with ice-cold phosphate-buffered saline (PBS) and then lysed with buffer (50 mM Tris–Cl, 150 mM NaCl, 100 μg/ml phenylmethylsulfonyl fluoride, protease and phosphatase inhibitor cocktail from Roche, and 1% Triton X-100) on ice for 30 min. After centrifugation at 12,000 *g* for 20 min at 4 °C, the supernatant was used for Western blot analysis as previous described. The primary antibodies used in this study are listed in Supplementary Table 1.

Aβ expression in the CL2006 strain was identified by immunoblotting on a Tris–tricine gel. Briefly, worms were collected and washed by M9 buffer and then transferred to the cell lysis buffer containing a protease inhibitor cocktail (Roche) for sonication. After centrifugation at 12,000 *g* for 20 min at 4 °C, the supernatant was used for Western blot analysis. Protein concentration was determined using the Bradford method. Equal amounts of protein (50 μg) were loaded and separated by Tris–tricine sodium dodecyl sulfate–polyacrylamide gel electrophoresis. After transfer, the polyvinylidene difluoride membrane (Millipore, 0.2 μm) was boiled 5 min in PBS buffer and blocked in Tris-buffered saline/Tween 20 containing 5% nonfat milk for 1 h, then it was processed as a regular Western blot.

Gene expression analysis with quantitative real-time PCR

Total RNA was extracted from adult worms using Trizol reagent (Invitrogen), and cDNA was synthesized with the SuperScript III First-Strand Synthesis Super-Mix for quantitative PCR (Invitrogen). The primers used are listed in Supplementary Table 2. The ama-1 gene was used as the internal control. Quantitative PCR was carried out in a Rotor-Gene 6000 centrifugal real-time cycler (Corbett Research, Qiagen, Valencia, CA, USA) using the Platinum SYBR Green qPCR SuperMix-UDG with ROX kit. The cycling conditions were as

follows: initial denaturation at 95 °C for 2 min, followed by 45 cycles of 15 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C.

Statistical analysis

All values are expressed as the mean ± standard error. Statistical significance was defined as $p < 0.05$. One-way or two-way analysis of variance (ANOVA) was used to test each variable for differences among the treatment groups with StatView (SAS Institute, Inc.). If ANOVA demonstrated a significant effect, pairwise post hoc comparisons were made with Fisher's least significant difference test.

Results

ADMA was increased in Aβ-transgenic C. elegans and APPsw cells

To determine whether Aβ expression could increase ADMA production, we measured ADMA levels in the wild-type (N2) and Aβ transgenic *C. elegans* (CL2006). On the first day of adulthood, the ADMA level of CL2006 worms was ~25% higher than that of the N2 worms (0.70 ± 0.12 vs 0.55 ± 0.12 mg/g), although the difference was not statistically significant yet. On the 12th day of adulthood, the ADMA level was almost doubled in the CL2006 worms and was statistically significantly higher than that of the N2 worms (1.31 ± 0.06 vs 0.65 ± 0.04 mg/g, $n=3$, $p < 0.05$; Fig. 1A).

We then examined the ADMA level in the APPsw SH-SY5Y cells and the control *neo* cells. As expected, the ADMA level in APPsw cell lysate was 50% higher than in that of *neo* cells (Fig. 1B). Associated with increased ADMA level, higher PRMT1 levels were observed in the CL2006 worms and APPsw cells (Fig. 1C–E), indicating that PRMT1 upregulation may contribute to the enhanced ADMA accumulation. The expression levels of DDAH1 and DDAH2 were also significantly increased in the APPsw cells, which might be a compensatory response to the elevated ADMA level. As NO is believed to be directly or indirectly involved in AD pathology, we compared NOS expression in the *neo* and APPsw cells. Expression of both inducible NOS (iNOS) and neuronal NOS (nNOS) was significantly higher in the APPsw cells (Fig. 1D and E), which is in agreement with a previous report that the APPsw cells have a higher intracellular NO level [23]. Endothelial NOS (eNOS) was almost undetectable in the *neo* and APPsw cells.

ADMA exacerbates Aβ-induced paralysis and oxidative stress in CL2006 worms

In the CL2006 worms, human $A\beta_{1-42}$ protein is expressed in the body wall muscle cells. The expression and subsequent aggregation of Aβ in the muscle lead to progressive paralysis of these worms [20]. To investigate whether ADMA specifically affects Aβ-induced toxicity in vivo, we fed CL2006 worms with 1 μM ADMA for 12 days and then examined the paralysis of the worms. We found that ADMA significantly increased paralysis of the CL2006 worms (54.31 ± 6.29% vs 37.43 ± 4.08%, $n=3$, $p < 0.05$) but had no effect on the N2 worms (12.51 ± 5.35% vs 13.66 ± 3.04%; Fig. 2A).

Given that oxidative stress has emerged as one of the important factors in AD pathogenesis, we examined the effects of ADMA on Aβ-induced intracellular H_2O_2-associated ROS in *C. elegans*. ADMA treatment resulted in a significantly greater increase in ROS levels in the CL2006 worms compared to the N2 worms on the 12th day of adulthood (Fig. 2B and C), suggesting ADMA causes more oxidative stress in CL2006 worms.

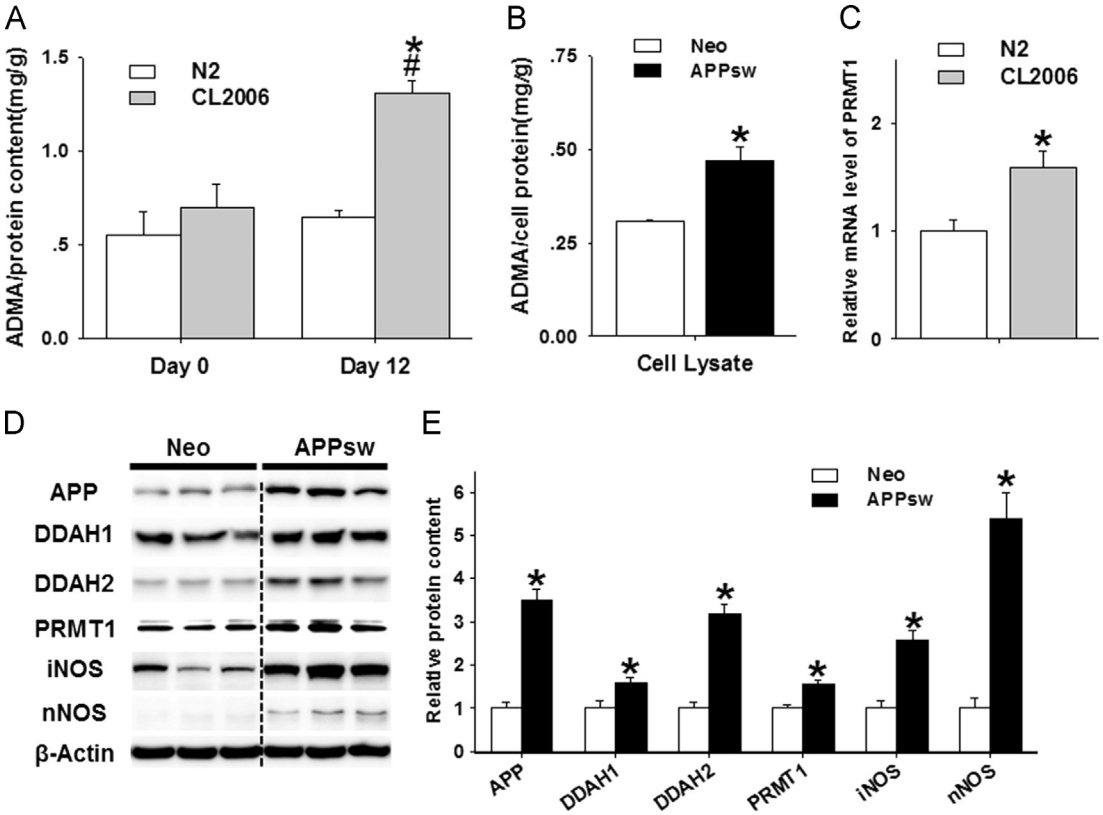

Fig. 1. ADMA accumulation in CL2006 worms and APPsw cells. ELISA was used to determine ADMA level (A) in the N2 and CL2006 worms on the first and 12th day of adulthood, as well as (B) in the *neo* and APPsw cells. (C) PRMT1 mRNA level in *C. elegans* on the 12th day of adulthood was examined by real-time RT-PCR. (D, E) The protein levels of amyloid-β precursor protein (APP), DDAH1, DDAH2, PRMT1, iNOS, and nNOS were determined by Western blot. β-Actin was used as a loading control. Results were collected from three to six independent experiments. *$p < 0.05$ compared with the N2 worms or to the *neo* cells. #$p < 0.05$ compared to the first day of adulthood.

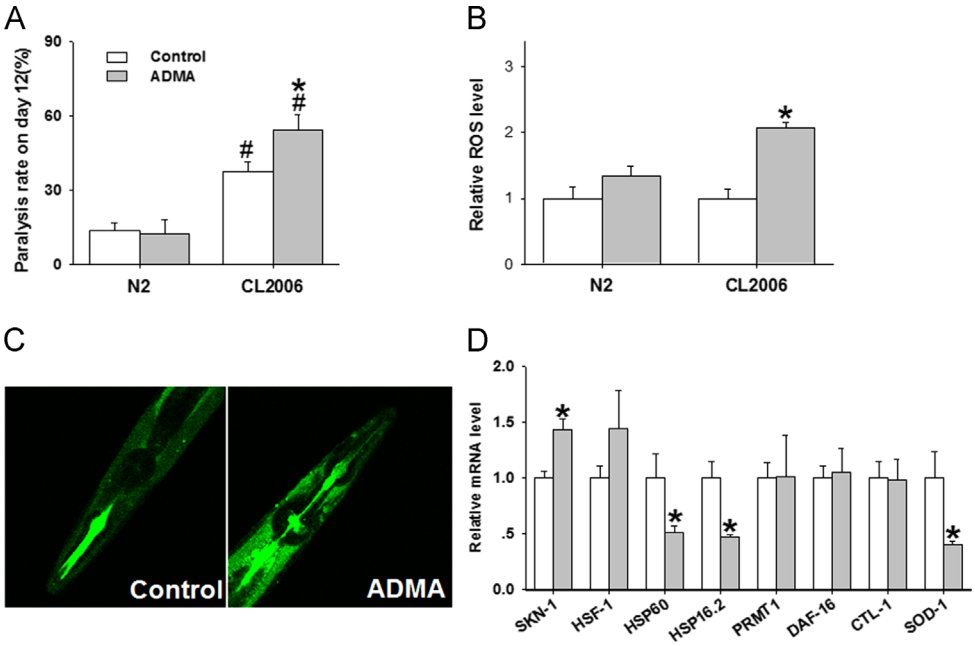

Fig. 2. ADMA aggravated Aβ-induced paralysis and oxidative stress in *C. elegans*. (A) On the 12th day of adulthood, paralysis was determined in the N2 and CL2006 worms. (B) The ROS level in *C. elegans* was measured with the DCFH-DA method. (C) The DCF fluorescence in control and ADMA-treated worms was imaged using confocal microscopy. (D) The mRNA levels of oxidative stress-related genes were examined in control and ADMA-treated CL2006 worms by real-time RT-PCR. Results were collected from three to six independent experiments. $^{*}p < 0.05$ compared to the control worms. $\#p < 0.05$ compared to the N2 worms.

The SKN-1 transcription factor is a functional ortholog of the mammalian protein Nrf2 (nuclear factor-E2-related factor 2) in *C. elegans* [24]. It can be induced by oxidative stress. Consistent with the increase in ROS, the mRNA level of SKN-1 was significantly increased in the ADMA-treated CL2006 worms. ADMA had no effect on protective genes such as HSF-1 (heat shock transcription factor 1), DAF-16 (a forkhead transcription factor), and CTL-1 (catalase), but significantly repressed the expression of heat shock protein 60 (HSP60), small heat shock protein 16.2 (HSP16.2), and superoxide dismutase 1 (SOD-1) (Fig. 2D).

ADMA promotes Aβ aggregation

As the relationship between Aβ expression/aggregation and paralysis behavior has been established in the CL2006 worms, we determined the Aβ expression using Western blot. As shown in Fig. 3, ADMA treatment significantly increased the level of toxic Aβ oligomers, suggesting more Aβ aggregation in the ADMA-treated CL2006 worms. There was also a trend toward increased Aβ monomer after ADMA treatment, but the difference was not significant.

Knockdown of PRMT1 exacerbates Aβ-induced paralysis in CL2006 worms

As PRMT1 is the major type I arginine methyltransferase for ADMA production in *C. elegans* [25], we wanted to determine whether decreasing ADMA production by knockdown of PRMT1 could protect against Aβ toxicity in *C. elegans*. Surprisingly, knockdown of PRMT1 significantly increased the paralysis in the CL2006 worms on day 12 of adulthood compared with control RNAi groups (Fig. 4A), but had no effect on the N2 worms (data not shown). PRMT1 knockdown did not affect ROS levels (Fig. 4B), but caused significant reductions in HSP60 and SOD-1 mRNA levels in the CL2006 worms (Fig. 4C).

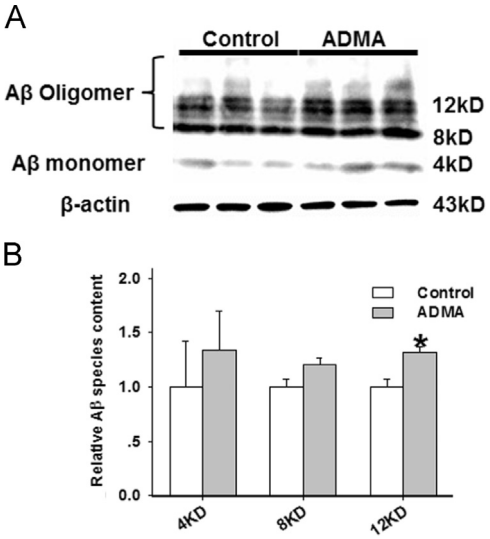

Fig. 3. ADMA promotes Aβ aggregation in CL2006 worms. (A) Representative Western blot of Aβ species in transgenic *C. elegans* CL2006 fed with or without ADMA. (B) Quantification of Aβ oligomers (~12 and ~8 kDa) and monomers (~4 kDa) from Western blot analysis. $^{*}p < 0.05$ compared to the control worms.

ADMA increased oxidative stress in APPsw cells

To further investigate the role of ADMA in the pathogenesis of AD, we selected APPsw cells as an in vitro model. Treatment with ADMA (20–60 μM) for 72 h caused a dose-dependent decrease in APPsw cell viability, especially at 40 μM (Supplementary Fig. 1). Next, we treated the APPsw cells with 40 μM ADMA for 24 h and measured the intracellular ROS and NO levels. ADMA incubation increased intracellular ROS level by 42% (Fig. 5A). The ratio of reduced glutathione (GSH) to oxidized glutathione (GSSG), a marker of oxidative stress, was decreased by 39% in the presence of ADMA (Fig. 5B). Because ADMA is a potent NOS inhibitor, we were surprised to observe that intracellular NO levels were not reduced by ADMA treatment in the APPsw cells (Fig. 5C). However, this observation may be explained by the finding

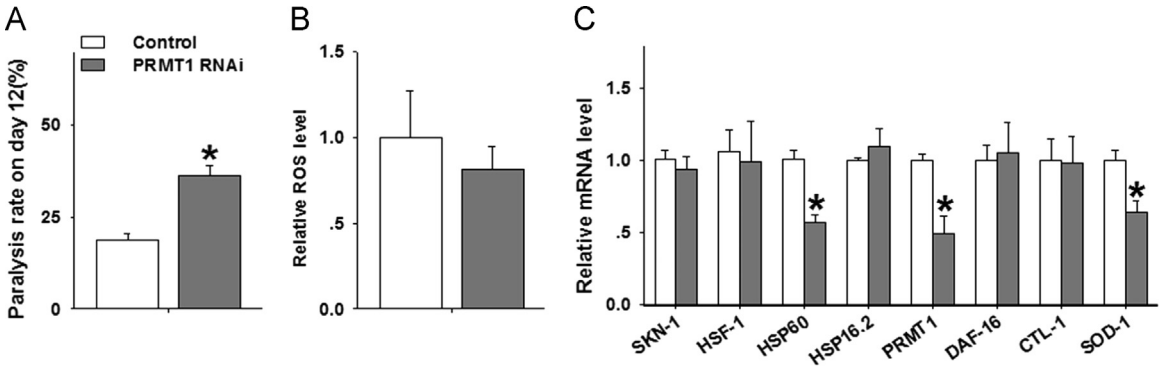

Fig. 4. Effects of PRMT1 knockdown on Aβ-induced paralysis and oxidative stress in CL2006 worm. CL2006 worms were maintained on the RNAi plates and on the 12th day of (A) adulthood paralysis and (B) ROS levels were determined. (C) The mRNA levels of oxidative stress-related genes were examined in the control and PRMT1-knockdown CL2006 worms by real-time RT-PCR. Results were collected from three to six independent experiments. $^{*}p < 0.05$ compared to control worms.

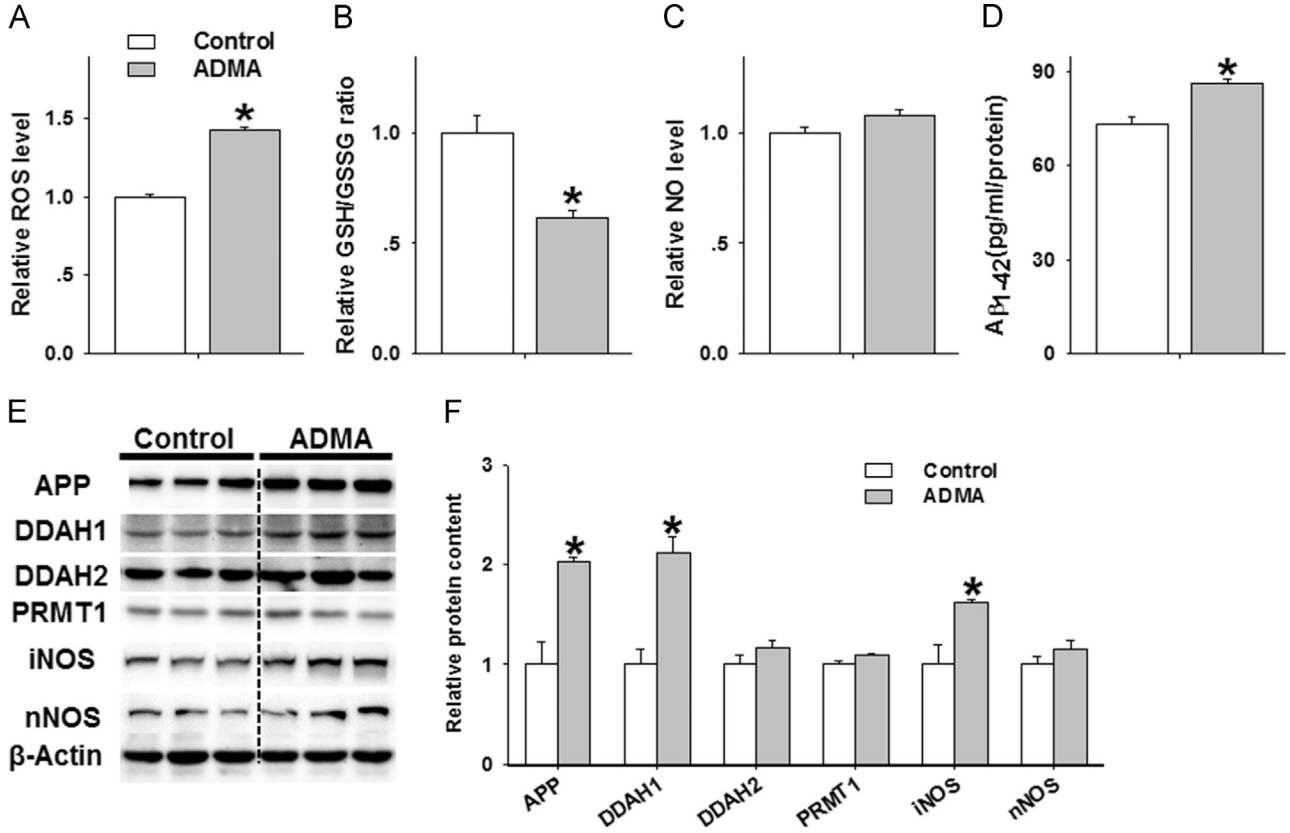

Fig. 5. ADMA exacerbated Aβ-induced oxidative stress in APPsw cells. After 24 h incubation with 40 μM ADMA, (A) intracellular ROS, (B) ratio of GSH/GSSG, (C) intracellular NO level, and (D) $A\beta_{1-42}$ level in the medium were determined ($n=6$). (E, F) The expression of APP, DDAH1, DDAH2, PRMT1, iNOS, and nNOS was determined by Western blot. β-Actin was used as a loading control. $^{*}p < 0.05$ compared to vehicle-treated APPsw cells.

that ADMA caused a significant increase in iNOS, which constitutively produces NO (Fig. 5E and F). To determine the effect of ADMA on Aβ secretion, the levels of $A\beta_{1-42}$ in the culture medium were measured using a sandwich ELISA kit. The results showed that ADMA administration increased the $A\beta_{1-42}$ secretion by 16.7% (Fig. 5D). Western blot revealed that ADMA treatment significantly increased expression of APP, DDAH1, and iNOS, but had no effect on DDAH2, PRMT1, and nNOS expression (Fig. 5E and F). These findings imply that ADMA may contribute to AD development.

Knockdown of PRMT1 exacerbates oxidative stress and Aβ secretion in APPsw cells

In mammalian cells, PRMT1 is the dominant type I arginine methyltransferase that generates ADMA, whereas PRMT5 is the primary type II arginine methyltransferase that generates symmetric dimethylarginine mark [26]. Because PRMT1 knockdown exacerbates Aβ-induced paralysis in the CL2006 worms, we also performed RNAi experiments in APPsw cells to decrease PRMT1 expression. The knockdown efficiency was confirmed by Western blot. In comparison with control siRNA, PRMT1 siRNA transfection resulted in significant increases in both intracellular ROS and NO levels, as well as a significant decline in the ratio of GSH/GSSG in the APPsw cells (Fig. 6A–C). Consistent with the altered redox state, more Aβ secretion was found in the medium of the cells with PRMT1 knocked down (Fig. 6D). As shown in Fig. 6E and F, knockdown of PRMT1 had no effect on APP, DDAH1, DDAH2, and PRMT5 expression, but significantly increased iNOS and nNOS expression, which might contribute to the increased intracellular NO level.

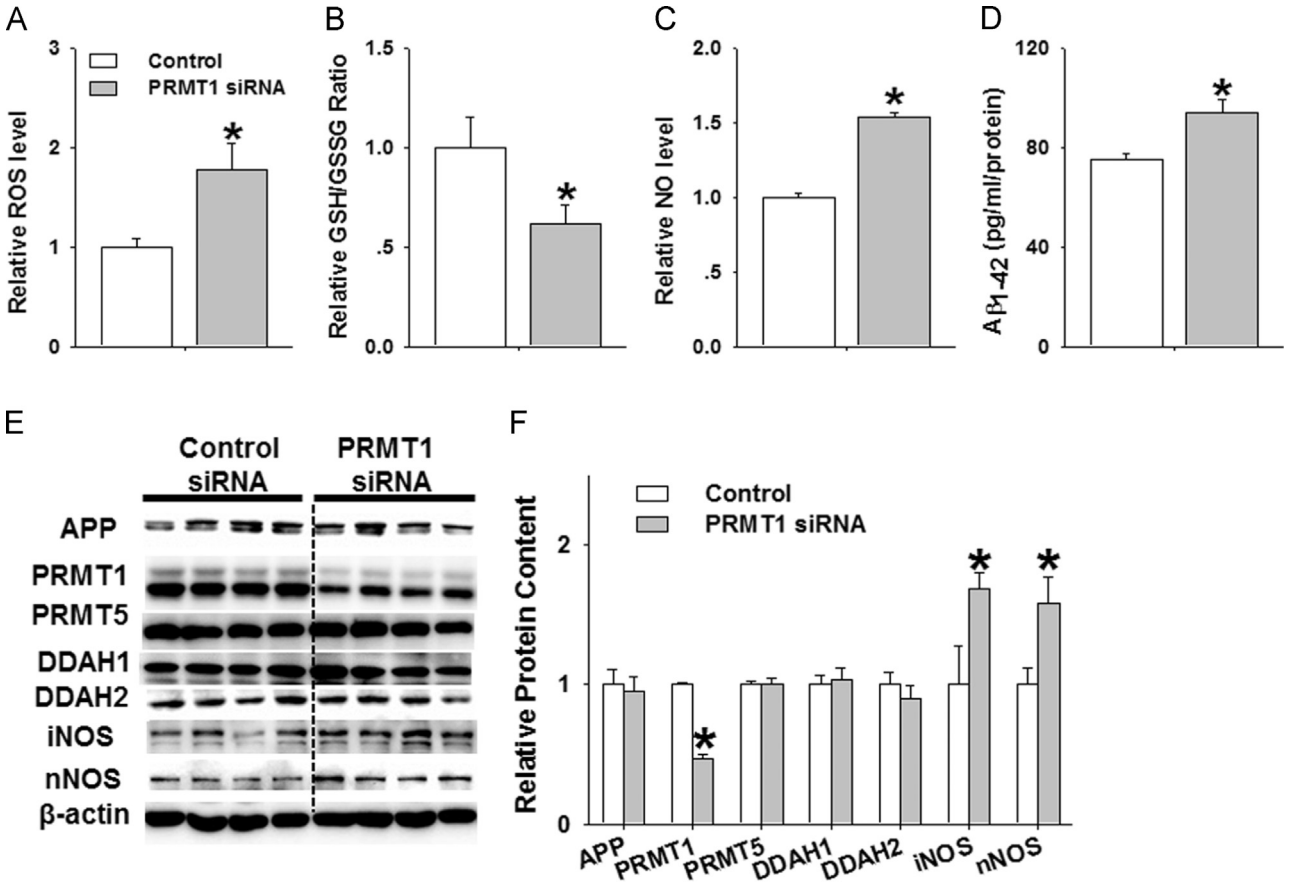

Fig. 6. Effects of PRMT1 knockdown on Aβ-induced oxidative stress in APPsw cells. After transfection with control or PRMT1 siRNA for 72 h, (A) intracellular ROS, (B) ratio of GSH/GSSG, (C) intracellular NO level, and (D) $A\beta_{1-42}$ level in the culture medium were determined ($n=6$). (E, F) Lysates of control and PRMT1 siRNA-transfected APPsw cells were examined by Western blot for the expression of APP, PRMT1, PRMT5, DDAH1, DDAH2, iNOS, and nNOS. β-Actin was used as a loading control. $^*p < 0.05$ compared with the control siRNA-transfected APPsw cells.

Overexpression of DDAH1 decreased oxidative stress and Aβ secretion in APPsw cells

DDAH1 plays a critical role in intracellular ADMA degradation [27]. Because decreasing ADMA production by knockdown of PRMT1 failed to show beneficial effects, we examined whether increasing the degradation of endogenous ADMA could decrease Aβ secretion. To accomplish this, APPsw cells were infected with DDAH1-expressing adenovirus or green fluorescent protein (GFP)-expressing adenovirus as a control. The efficiency of adenovirus infection in the APPsw cells was verified by fluorescence images (Supplementary Fig. 2) and Western blot (Fig. 7E). DDAH1 overexpression caused a 30% reduction in extracellular ADMA (data not shown). Because the fluorescent probes DCFH and DAF-FM were not suitable for intracellular ROS and NO detection in cells with GFP fluorescence, we used dihydroethidium and a total nitric oxide assay kit to measure intracellular $O_2^{\bullet -}$ and NO_x, respectively. DDAH1 overexpression resulted in ~20% reduction in the intracellular $O_2^{\bullet -}$ and ~24% increase in the ratio of GSH to GSSG (Fig. 7A and B), suggesting DDAH1 overexpression ameliorated oxidative stress in the APPsw cells. Surprisingly, DDAH1 overexpression had no effect on the NO_x level in the culture medium (Fig. 7C). Importantly, DDAH1 adenovirus infection reduced Aβ secretion by 36% (80.4 ± 6.0 vs 51.7 ± 3.7 pg/ml, normalized to total protein levels; Fig. 7D). APP expression was also decreased, whereas iNOS expression was increased in DDAH1-overexpressing cells. There were no significant changes in DDAH2, PRMT1, or nNOS expression after DDAH1 overexpression (Fig. 7E and F).

Knockdown of DDAH1 exacerbates oxidative stress and Aβ secretion in APPsw cells

To further investigate the protective role of DDAH1, we transfected APPsw cells with control or DDAH1-specific shRNA-expressing vector, which repressed DDAH1 expression by 75%, but had no effect on DDAH2, PRMT1, or nNOS expression (Fig. 8E and F). Knockdown of DDAH1 increased the intracellular ROS level by 49% and decreased the GSH/GSSG ratio by 18% (Fig. 8A and B). DDAH1 knockdown also significantly increased the APP expression and Aβ secretion (Fig. 8D–F). Although the iNOS expression was 20% higher in the DDAH1-knockdown cells, the intracellular NO level was still the same as that of the control cells (Fig. 8C, E, and F).

PRMT1–ADMA–DDAH1 modulate mitogen-activated protein kinase (MAPK) signaling in APPsw cells

Aβ has been reported to induce the phosphorylation of p38 MAPK and extracellular signal-regulated kinase (ERK), whereas inhibition of p38 or ERK is known to decrease Aβ secretion in the APPsw cells [28]. We found that the levels of phosphorylated $p38^{Thr180/Tyr182}$ and $ERK^{Thr202/Tyr204}$ were significantly increased in APPsw cells compared with *neo* cells and that ADMA treatment or knockdown of PRMT1 further increased $p38^{Thr180/Tyr182}$ and $ERK^{Thr202/Tyr204}$ phosphorylation in APPsw cells (Fig. 9A–C and E). DDAH1 overexpression or knockdown did not affect $p38^{Thr180/Tyr182}$ phosphorylation. However, overexpression of DDAH1 did

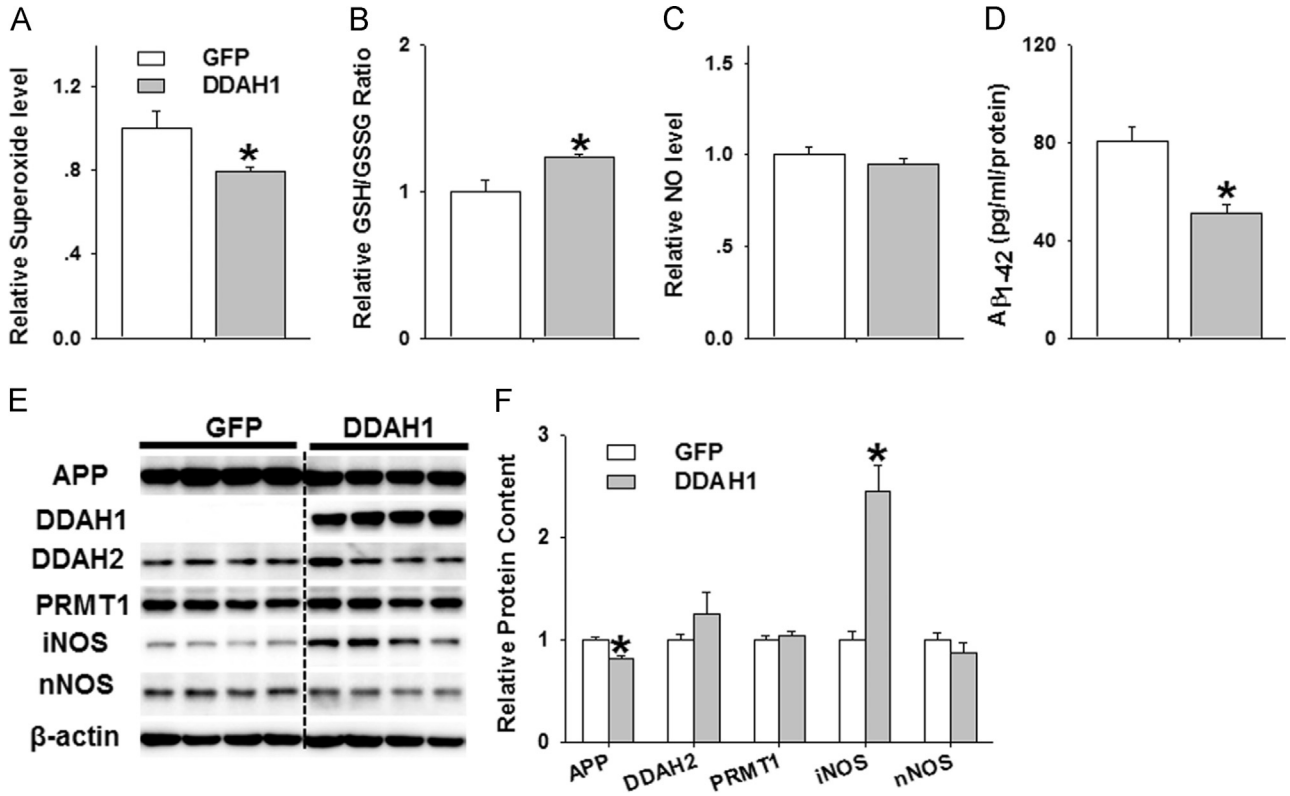

Fig. 7. Effects of DDAH1 overexpression on Aβ-induced oxidative stress in APPsw cells. After infection with GFP or DDAH1 adenovirus for 72 h, (A) intracellular ROS, (B) ratio of GSH/GSSG, (C) intracellular NO level, and (D) $A\beta_{1-42}$ level in the cell medium were determined ($n=6$). (E, F) Lysates of GFP and DDAH1 adenovirus-infected APPsw cells were examined by Western blot for expression of APP, DDAH1, DDAH2, PRMT1, iNOS, and nNOS. β-Actin was used as a loading control. $^{*}p < 0.05$ compared to the APPsw cells infected with GFP adenovirus.

significantly decrease $ERK^{Thr202/Tyr204}$ phosphorylation. Conversely, DDAH1 knockdown increased $ERK^{Thr202/Tyr204}$ phosphorylation (Fig. 9D and F, Supplementary Fig. 3A and B), suggesting that DDAH1 may exert a protective effect in this AD model in part by inhibiting ERK activity.

Discussion

The major new finding of this study is that ADMA levels are increased in the AD models and that exogenous ADMA can exacerbate Aβ-induced oxidative stress. These findings imply that ADMA is both a biomarker of AD and a contributing factor in AD development. To our knowledge, these findings provide the first direct evidence that the PRMT1–ADMA–DDAH1 axis plays a role in the pathophysiological mechanisms of AD.

Recent studies have demonstrated that NO availability may be a common link between cardiovascular risk factors and the development of AD. For example, reduced eNOS activity and NO level resulted in higher expression of APP, β-site APP-cleaving enzyme 1, and Aβ production in an animal model [3]. Unlike NO, ADMA concentrations are relatively stable and can be accurately measured in plasma. There is good evidence that the plasma ADMA levels are elevated in the AD patients [5,6,18] and higher plasma ADMA concentration is believed to contribute to AD development by attenuating vascular NO production and interfering with cerebral microperfusion [5]. Here we demonstrated that increasing Aβ expression in the CL2006 worms or APPsw cells resulted in more ADMA production. In addition, we also found that administration of ADMA has no effect on the age-related behavior of the N2 worms, but significantly exacerbates Aβ-induced paralysis in the CL2006 worms. As the paralysis behavior was associated with Aβ expression and aggregation in the CL2006 worms, we examined whether ADMA might affect paralysis by modulating Aβ expression/aggregation. Indeed, Western blot analysis demonstrated that ADMA significantly increased the levels of the toxic Aβ oligomers. In the APPsw cells, ADMA treatment caused more APP expression and Aβ secretion. Together, these data indicate that chronic ADMA accumulation may exacerbate AD pathology.

Although the most obvious consequence of increased ADMA levels is inhibited NO production, recent reports indicate that ADMA may cause NOS to generate $O_2^{\bullet-}$ rather than NO. Several in vitro studies have demonstrated that the addition of ADMA caused $O_2^{\bullet-}$ generation by purified NOS protein [29], in cultured human endothelial cells [30], in isolated arterioles from rat gracilis muscle [31], and in a murine lung epithelial cell line, LA-4 [32]. In addition, increased plasma ADMA was associated with elevated vascular superoxide production and eNOS uncoupling in patients with advanced atherosclerosis [33]. ADMA may also directly upregulate the renin–angiotensin system via a mechanism independent of NOS action and subsequently increase ROS production [34]. Although there is no NOS enzymes in *C. elegans* [35], ADMA addition still significantly increased ROS in the CL2006 worms, which subsequently induced SKN-1, a protein that controls the induction of multiple genes involved in detoxification and antioxidant stress response in *C. elegans* [24]. Because activation of heat shock proteins (especially HSP16.2) reduces Aβ toxicity in a transgenic *C. elegans* model of AD (strain CL4176) by diminishing oligomers and decreasing ROS level [36], we speculate that the downregulation of HSP60, HSP16.2, and SOD-1 mRNA levels in response to ADMA might be responsible for the increase in ROS. The limitation of this study is that we could not further determine exactly which pathway is responsible for the increase in ROS in

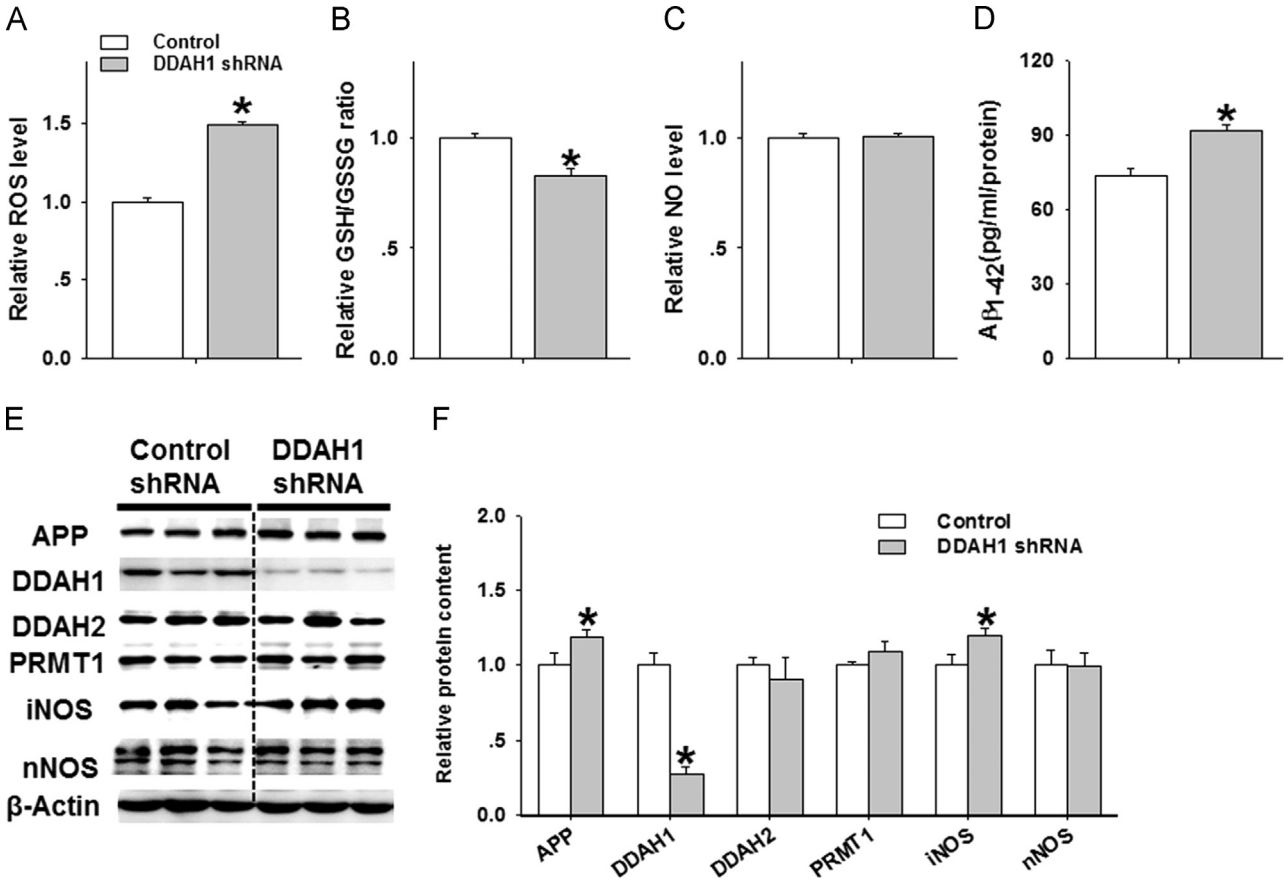

Fig. 8. Effects of DDAH1 knockdown on Aβ-induced oxidative stress in APPsw cells. After transfection with control or DDAH1 shRNA for 72 h, (A) intracellular ROS, (B) ratio of GSH/GSSG, (C) intracellular NO level, and (D) $A\beta_{1-42}$ level in the cell medium were determined ($n=6$). (E, F) Lysates of control and DDAH1 shRNA-transfected APPsw cells were examined by Western blot for expression of APP, DDAH1, DDAH2, PRMT1, iNOS, and nNOS. β-Actin was used as a loading control. *$p < 0.05$ compared to the control shRNA-transfected APPsw cells.

ADMA-treated worms, as we do not have the relevant mutant strains in hand.

Although ADMA can increase ROS, ROS can also stimulate ADMA production and/or inhibit ADMA degradation, which may further increase ADMA concentration in a positive feedback fashion. Hyperglycemia-induced ROS production may elevate PRMT-1 expression and increase ADMA levels. We also observed that the CL2006 worms and the APPsw cells have higher levels of PRMT1, suggesting that PRMT1 is a redox-sensitive enzyme. However, knockdown of PRMT1 failed to decrease oxidative stress in the CL2006 worms and the APPsw cells. By contrast, PRMT1 RNAi exacerbated the paralysis in the CL2006 worms and caused more oxidative stress and Aβ secretion in the APPsw cells. Although no previous reports have directly examined the effect of PRMT1 on Aβ-induced oxidative stress, there is evidence that PRMT1 can affect cellular oxidative stress through posttranslational modification of target proteins. For example, PRMT1 knockdown inhibited Nrf2 binding to the ARE by ~40% [37]. Thus, PRMT1 blockade in the development of AD is detrimental.

ADMA is mainly metabolized by DDAH, which is an extremely oxidant-sensitive enzyme [38]. Immunostaining results showed that DDAH is specifically elevated in neurons displaying cytoskeletal abnormalities and oxidative stress in AD patients, whereas it was undetectable in the neurons of age-matched healthy controls [39]. Consistent with such an observation, we found that expression of DDAH1 and DDAH2 was significantly increased in APPsw cells. This might be a compensatory response to ADMA accumulation as ADMA incubation further increased DDAH1 expression. It was reported that reduced DDAH activity, but not expression, is responsible for serum ADMA elevation in heart failure [40], hypercholesterolemia [41], and diabetes mellitus [42]. As DDAH is a monomeric enzyme containing one noncatalytic zinc, its activity could be competitively inhibited by low concentrations of free zinc ($K_i=2.0$ μM) [43]. The abnormalities in zinc metabolism might result in decreased DDAH activity in AD [39]. Homocysteine, a risk factor for AD and cognitive dysfunction in normal aging, also inactivates DDAH in neurons by reacting with the cysteine residue in its active site, causing the accumulation of ADMA and the inhibition of NO synthesis [44]. Although there are two isoforms of DDAH, recent studies demonstrated that DDAH1 is the essential enzyme for ADMA degradation in mammalian cells [27]. In the present study, we found that overexpression of DDAH1 significantly decreased oxidative stress and Aβ secretion in APPsw cells, whereas knockdown of endogenous DDAH1 exacerbated cellular oxidative stress and Aβ secretion. These data suggest that DDAH1 exerts a pivotal protective role in AD development.

$A\beta_{1-42}$-induced oxidative stress is known to activate ERK and p38 MAPK in AD. Activated MAPK signaling pathways are thought to contribute to AD pathogenesis through various mechanisms, including induction of neuronal apoptosis and transcriptional/enzymatic activation of β- and γ-secretases [45]. The finding that ADMA treatment increased phosphorylation of p38 and ERK in human endothelial cells [46] is consistent with our observation that ADMA exacerbated Aβ-induced increase in $p38^{Thr180/Tyr182}$ and $ERK^{Thr202/Tyr204}$ phosphorylation. The regulation of ERK activity by DDAH1 overexpression or knockdown was also observed in endothelial cells [47], whereas the activation of MAPK signaling by PRMT1 knockdown has not been previously reported.

In summary, our study demonstrates for the first time that ADMA accumulates in the development of AD and that elevated ADMA level further exacerbates AD progression through elevated

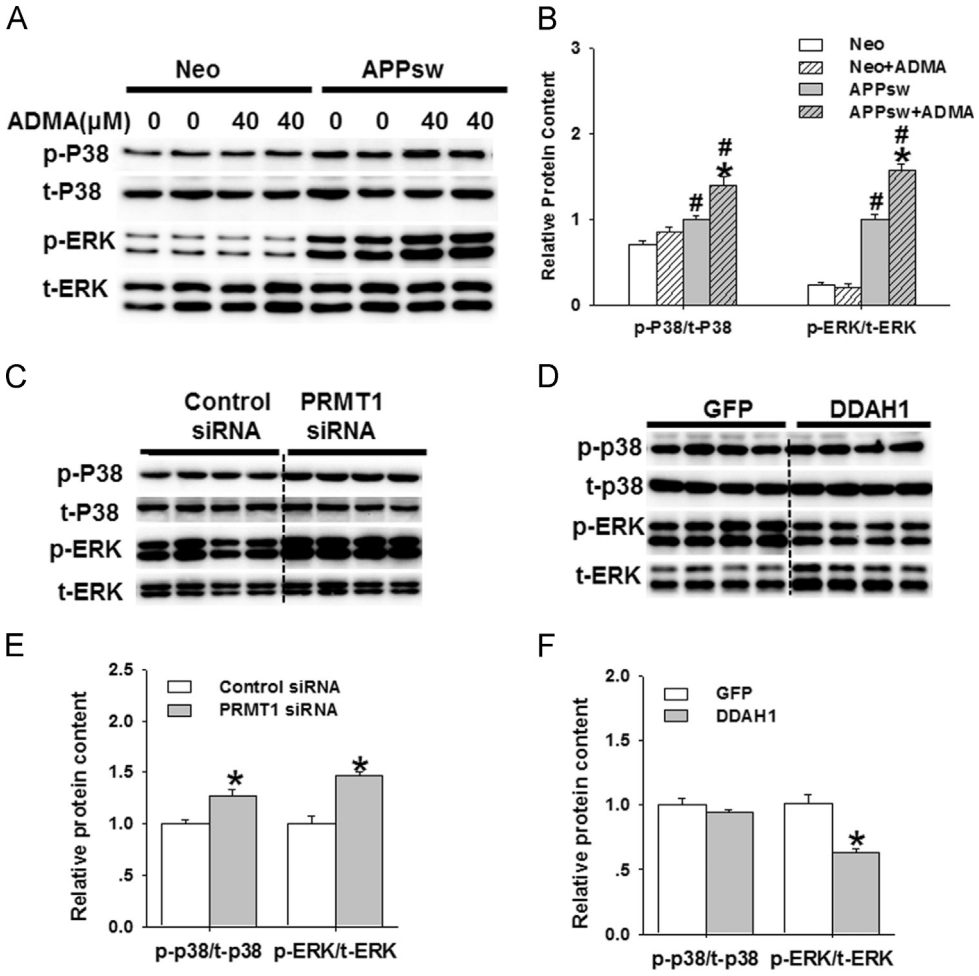

Fig. 9. Effects of ADMA, PRMT1, and DDAH1 on MAPK signaling. The phosphorylated and total p38 and ERK were measured in the (A, B) *neo* and APPsw cells with or without ADMA treatment, (C, E) control or PRMT1 siRNA-transfected APPsw cells, and (D, F) GFP or DDAH1 adenovirus-infected APPsw cells. $n=4$, $^{*}p<0.05$ compared to vehicle-treated, control siRNA-transfected, or GFP adenovirus-infected APPsw cells. $\#p<0.05$ compared to the *neo* cells.

ROS production. Our findings suggest that preservation of DDAH activity and the reduction of ADMA accumulation in neurons may be a new strategy for the treatment of Alzheimer disease.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.freeradbiomed.2014.12.002.

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